Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Supplementary Material

Comparative Epidemiology of Pandemic and Seasonal Influenza A in Households

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In the sections below we provide additional technical details of the laboratory methods used in our study, followed by Appendix Tables 1-2 and Appendix Figures 1-4.

RT-PCR

Total nucleic acid was extracted from specimens by using the NucliSens easyMAG extraction system (bioMerieux, the Netherlands) according to the manufacturer's instructions. Twelve microliters of extracted nucleic acid was used to prepare cDNA by using an Invitrogen Superscript III kit (Invitrogen, San Diego, California) with random primer, as described elsewhere.[1]

For detection of influenza A virus, 2μL of cDNA was amplified in a LightCycler 2.0 (Roche Diagnostics, Penzberg, Germany) with a total reaction-mix volume of 20μL reaction containing FastStart DNA Master SYBR Green I Mix reagent kit (Roche Diagnostics), 4.0mM MgCl2 and 0.5mM of each primer. The forward primer (5'-CTTCTAACCGAGGTCGAAACG-3') and the reverse primer (5'-GGCATTTTGGACAAAKCGTCTA-3) were used for amplification of the matrix gene of influenza A virus.[2] Cycling conditions were as follows: an initial denaturation at 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 10 seconds, 60ºC for 3 seconds, 72ºC for 12 seconds with ramp rates of 20ºC/second. At the end of the assay, PCR products were subjected to a melting curve analysis to determine the specificity of the assay.

For detection of influenza B virus, forward (5'- GCATCTTTGTTTTTTATCCATTCC) and reverse (5'-CACAATTGCCTACCTGCTTTCA) primers and 5' nuclease probe (Fam-TGCTAGTTCTGCTTTGCCTTCTCCATCTTCT-TAMRA) were used for amplification of the matrix gene.[3] Testing was performed using TaqMan EZ RT-PCR Core reagent kit (Applied Biosystems, Hammonton, New Jersey) comprising 0.8µmol/L of forward and reverse primers and 0.2µmol/L of probe in a total reaction volume of 25µL, comprising 4 µL of nucleic acid extract. Amplification and detection was performed on an ABI StepOne™ Real-Time PCR System (Applied Biosystems) with the following conditions: initial hold at 50ºC for 20 minutes and 95ºC for 15 minutes followed by 45 cycles at 95ºC for 15 seconds and 60ºC for 1 minute.

Viral culture

Madin-Darby canine kidney (MDCK) cell monolayers in culture tubes were inoculated with 200µL of the nasal swab-virus transport medium suspension and the cells were maintained in serum-free minimum essential medium (MEM) (Gibco, New York) containing tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (2µg/ml) (Sigma, St. Louis, Missouri), and incubated at 33ºC for 7 days (MDCK cells). They were examined daily for cytopathic effect, and immunofluoresence was done on fixed cell smears when CPE appeared or at the end of the incubation period.[2]

Quantitative virus culture was done using MDCK cells grown on microtitre plates. The cells were rinsed in serum-free MEM. The specimen was diluted initially by 1 in 5 and then in 10 fold steps in serum free MEM with 2ug/ml trypsin (see above). 100 ul of the undiluted specimen as well as each of the specimen dilutions was added in quadruplicate to the MDCK cell monolayers. Medium alone was added to control cells. An additional 100ul of serum free MEM with 2ug/ml trypsin was added to each well and the plates incubated at 33^0C for 7 days. The plates were examined for cytopathic effect daily. The TCID 50 was determined according to the Reed and Muench Method. The calculation is

= (log dilution above 50%) + (proportionate distance x log dilution factor)

$$\text{proportionate distance} = \frac{\text{\% positive above 50\% - 50\%}}{\text{(\% positive above 50\%) – (\% positive below 50\%)}}$$

A small number of specimens had insufficient biomaterial to permit testing by quantitative culture.

Serology

The sera were treated with receptor-destroying enzyme (RDE) (Denka Seiken Co Ltd, Tokyo) at 37°C overnight to remove nonspecific inhibitors and residual RDE was destroyed by heat inactivation at 56°C for 30 minutes. The hemagglutination inhibition (HAI) test was carried out in 96 well microtitre plates using reagents provided by World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza Melbourne or the WHO Collaborating Centre, Centres of Disease Control, Atlanta, GA using standard methods as detailed in the WHO reagent kit and elsewhere.[4] The viruses used were A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2) like virus A/Uruguay/716/2007 virus antigens provided as part of the World Health Organization (WHO) reagents. The pandemic H1N1 HA antigen was not included in the WHO reagent kit and was prepared by culture of A/California/4/2009 (H1N1) virus in MDCK cells. The recent A/Perth/16/2009 like (H3N2) virus was not included in HAI tests because of atypical reactions observed with this virus (personal communication, Alan Hay).

The conventional neutralization test for the A/California/4/2009 and the A/Perth/16/2009-like virus A/HK/1985/2009 was carried out in micro-titre plates using neutralization of virus cytopathogenic effect (CPE) in Madin-Darby Canine Kidney (MDCK) cells. Serial serum dilutions in quadruplicate were mixed with 100 tissue culture infectious dose 50 (TCID50) for 2 hours and added to MDCK cells. One hour after infection, serum-virus mixtures were removed and serum free MEM with 2 ug/ml trypsin was added to each well. The plates were incubated and cytopathic effect was observed to determine the highest serum dilution that neutralized ≥ 50% of the wells. A virus back titration and positive and negative control sera were included in each assay.

References

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Table 1. Characteristics of index cases and their household contacts.

Characteristic	**Index cases with pandemic influenza A virus infection**		**Index cases with seasonal influenza A virus infection**		p-value*
	n	(%)	n	(%)	
Index cases	**(n=45)**		**(n=54)**		
Age ≤5 years old	2	(4%)	1	(2%)	
6-15 years old	20	(44%)	19	(35%)	
16-30 years old	16	(36%)	10	(19%)	
31-50 years old	6	(13%)	13	(24%)	
>50 years old	1	(2%)	11	(20%)	0.01
Male sex	23	(51%)	29	(54%)	0.96
Signs and symptoms at recruitment:					
Temperature ≥37.8ºC	27	(60%)	43	(80%)	0.06
Headache	25	(56%)	35	(65%)	0.46
Sore throat	32	(71%)	41	(76%)	0.75
Cough	38	(84%)	45	(83%)	0.90
Myalgia	29	(64%)	36	(67%)	0.98
Runny nose	40	(89%)	48	(89%)	0.75
Phlegm	23	(51%)	25	(46%)	0.78
Diarrhea	1	(2%)	1	(2%)	1.00
Vomiting	1	(2%)	1	(2%)	1.00
Abdominal pain	1	(2%)	0	(0%)	0.45
Prescribed oseltamivir	23	(51%)	21	(39%)	0.31
Delay from illness onset to recruitment					
0-12 hours	6	(13%)	7	(13%)	
13-24 hours	15	(33%)	14	(26%)	
25-36 hours	9	(20%)	17	(31%)	
37-48 hours	15	(33%)	16	(30%)	0.61

Received influenza vaccine prior to 2008-09 season	9	(20%)	15	(28%)	0.51
Household contacts	**(n=130)**		**(n=154)**		
Age ≤5 years	4	(3%)	4	(3%)	
6-15 years	18	(14%)	31	(20%)	
16-30 years	19	(15%)	24	(16%)	
31-50 years	64	(49%)	62	(40%)	
>50 years	25	(19%)	33	(21%)	0.53
Male sex	51	(39%)	61	(40%)	0.95
Received influenza vaccine prior to 2008-09 season	18	(14%)	28	(18%)	0.41

* p-values calculated by chi-squared tests and Fisher exact tests

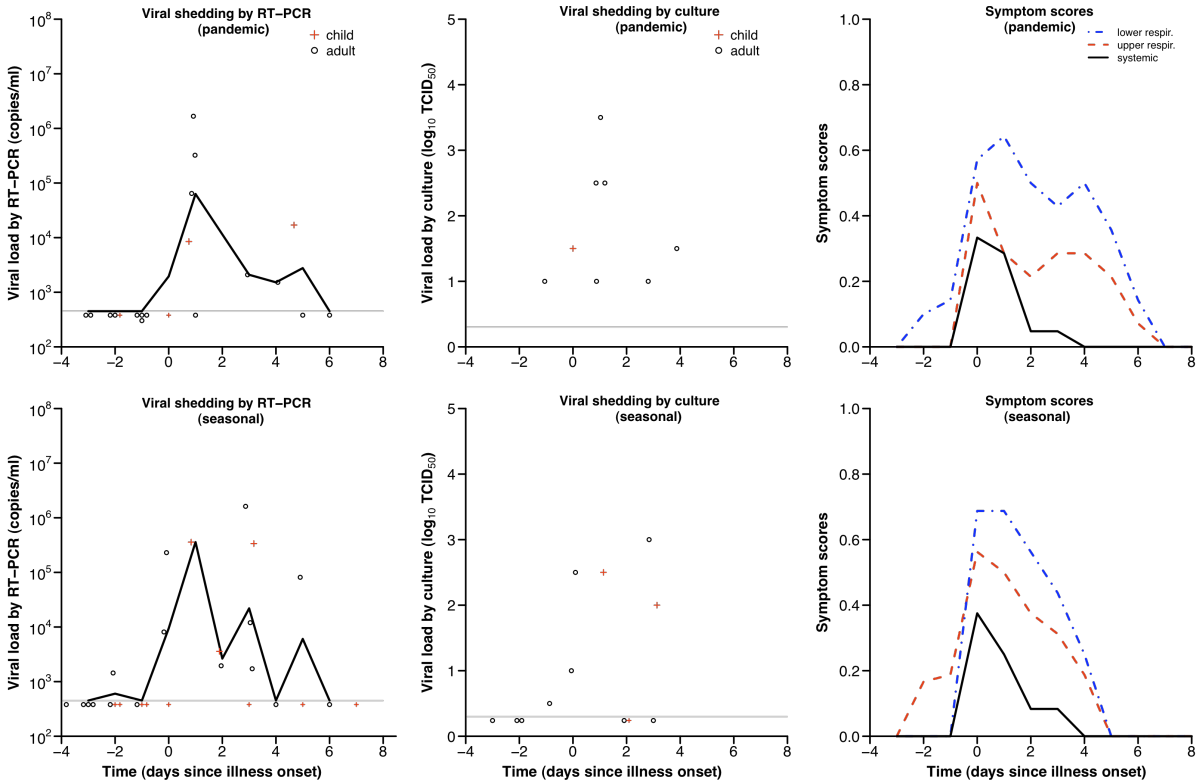
Appendix Table 2. Association between seroconversion, viral shedding and illness among 94 household contacts of index cases with pandemic and seasonal influenza A virus infection with acute and convalescent sera available.

Signs and symptoms during follow-up*	**Without 4-fold or greater rise in antibody titers†**		**With 4-fold or greater rise in antibody titers†**	
	No shedding by RT-PCR n (%)	Shedding by RT-PCR n (%)	No shedding by RT-PCR n (%)	Shedding by RT-PCR n (%)
Households of index cases with pandemic influenza A (H1N1)	(n=43)	(n=0)	(n=4)	(n=7)
Temperature ≥37.8ºC	1 (2%)	–	0 (0%)	3 (43%)
Cough	11 (26%)	–	0 (0%)	6 (86%)
ARI	11 (26%)	–	0 (0%)	6 (86%)
ILI	0 (0%)	–	0 (0%)	3 (43%)
Households of index cases with seasonal influenza A (H3N2)	(n=32)	(n=0)	(n=4)	(n=4)
Temperature ≥37.8ºC	0 (0%)	–	1 (25%)	2 (50%)
Cough	5 (16%)	–	2 (50%)	4 (100%)
ARI	4 (13%)	–	2 (50%)	4 (100%)
ILI	0 (0%)	–	0 (0%)	2 (50%)

*ARI is at least 2 of the following: temperature ≥37.8ºC, cough, headache, sore throat, myalgia, runny nose and phlegm; ILI is temperature ≥37.8ºC plus cough or sore throat. Therefore all individuals meeting the ILI definition also met the ARI definition.

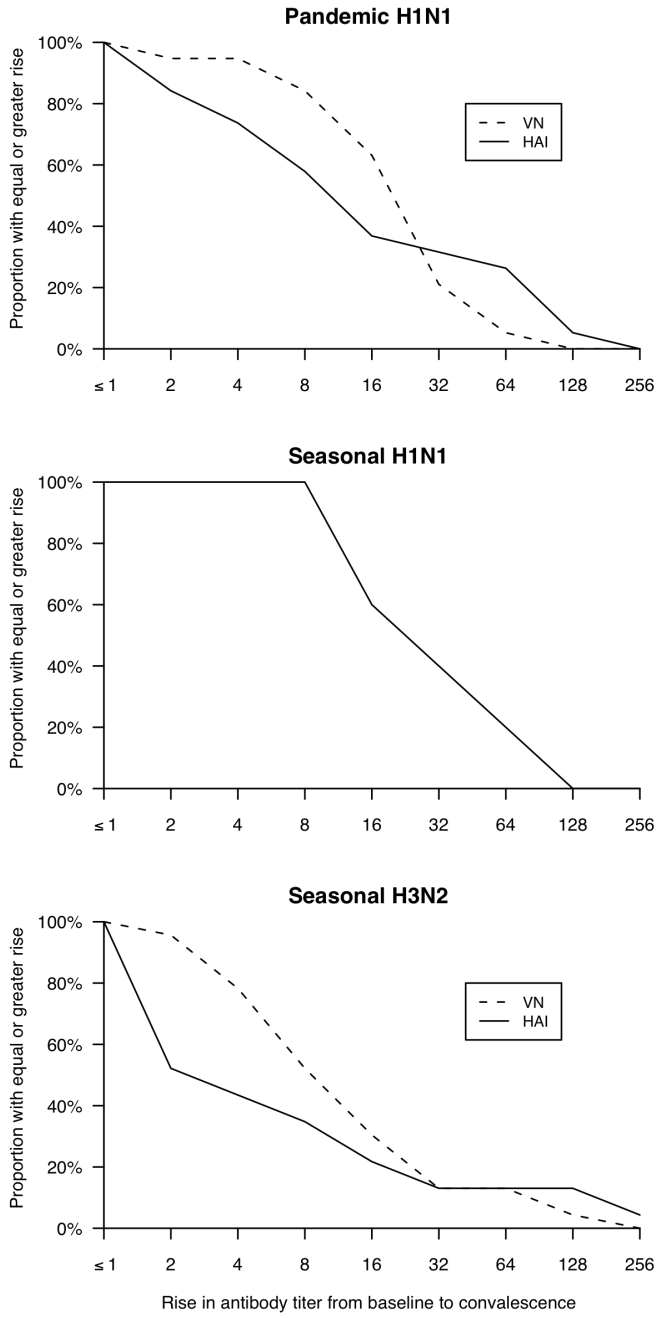
† Antibody titer by viral neutralization to A/California/4/2009 (H1N1) or A/Perth/16/09 (H3N2)-like virus A/HK/1985/2009, for household contacts of index cases with RT-PCR-confirmed pandemic H1N1 or seasonal H3N2 infection respectively.

Appendix Figure 1. Patterns of viral shedding and course of illness in secondary cases with RT-PCR-confirmed pandemic (top row) and seasonal (bottom row) influenza A virus infections by day relative to ARI onset* (day 0). First column: viral shedding for children (crosses) and adults (open circles), and the geometric mean viral shedding (solid lines) by RT-PCR. The lower limit of detection of the RT-PCR assay was approximately 900 copies/ml (grey line). Second column: tissue culture infectious dose ($TCID_{50}$) of specimens collected from children (crosses) and adults (open circles), and the geometric mean $TCID_{50}$. The lower limit of detection of the quantitative culture assay was approximately 100.3 $TCID_{50}$ (grey line). Third column: lower respiratory (dotted line), upper respiratory (dashed line) and systemic (solid line) symptom scores.

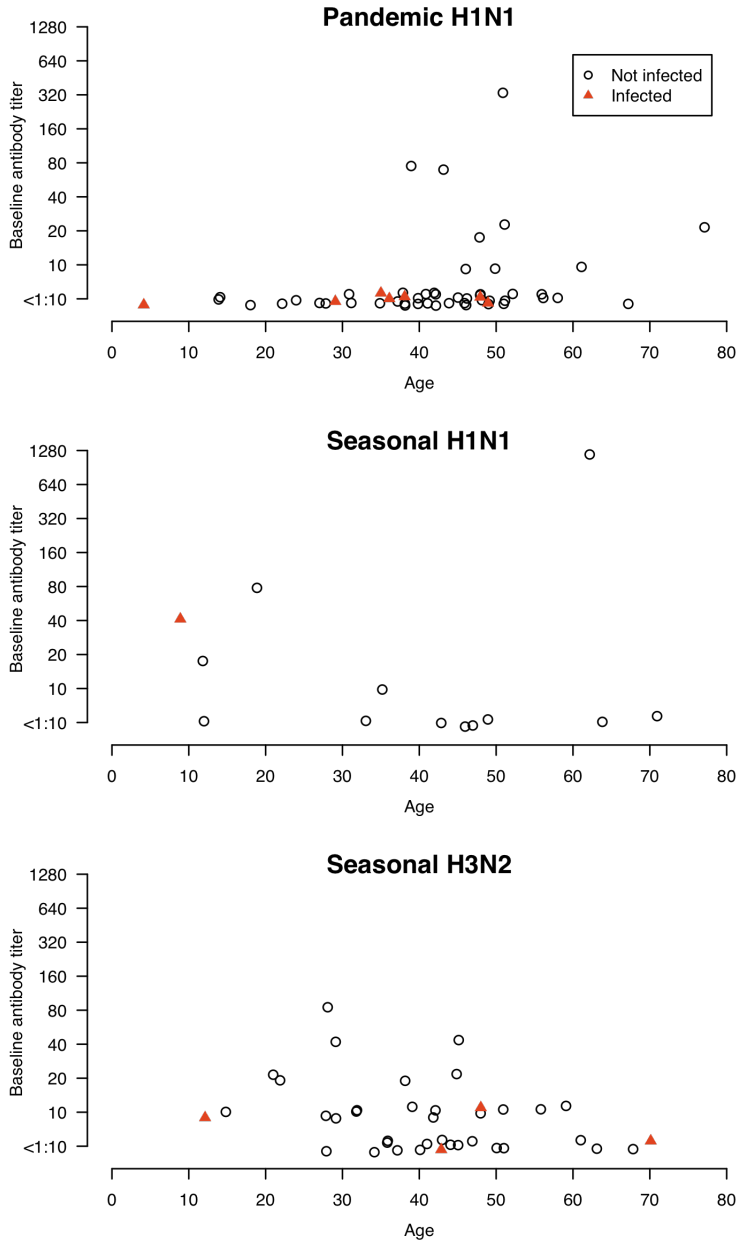


Footnote to Appendix Figure 1: ARI onset is defined as the first day with at least two of 7 self-reported signs or symptoms including fever≥37.8°C, cough, sore throat, myalgia, headache, runny nose or phlegm.

Appendix Figure 2. Top: Antibody titer rises from baseline to convalescence by HAI (solid line) and viral neutralization (dotted line) to A/California/4/2009 (H1N1) virus for 19 individuals with RT-PCR-confirmed pandemic influenza A (H1N1) virus infection. Center: Antibody titer rises from baseline to convalescence by HAI to A/Brisbane/59/2007-like (H1N1) virus for 5 individuals with RT-PCR-confirmed seasonal influenza A (H1N1) virus infection. Bottom: Antibody titer rises from baseline to convalescence by HAI (solid line) to A/Brisbane/10/2007 (H3N2)-like virus A/Uruguay/716/2007 and by viral neutralization (dotted line) to A/Perth/16/09 like virus A/HK/1985/2009 (H3N2) virus for 23 individuals with RT-PCR-confirmed seasonal influenza A (H3N2) virus infection.



Appendix Figure 3. Top: Baseline antibody titers by viral neutralization to A/California/4/2009 (H1N1) for 54 household contacts of index cases with RT-PCR-confirmed pandemic influenza A (H1N1) virus infection. Center: Baseline antibody titers by HAI to A/Brisbane/59/2007-like (H1N1) for 13 household contacts of index cases with RT-PCR-confirmed seasonal influenza A (H1N1) virus infection. Bottom: Baseline antibody responses by viral neutralization to A/Perth/16/09 like virus A/HK/1985/2009 (H3N2) for 40 household contacts of index cases with RT-PCR-confirmed seasonal influenza A (H3N2) virus infection. In each panel the household contacts with RT-PCR-confirmed infection during follow-up are indicated by triangles, other household contacts indicated by circles.



Appendix Figure 4. Top: Antibody titer rises from baseline to convalescence by HAI to A/California/4/2009 (H1N1), A/Brisbane/59/2007-like (H1N1) or A/Brisbane/10/2007 (H3N2)-like virus A/Uruguay/716/2007, for individuals with RT-PCR-confirmed pandemic H1N1 (left) or seasonal H3N2 infection (right). Bottom: Antibody titer rises from baseline to convalescence by viral neutralization to A/California/4/2009 (H1N1) or A/Perth/16/09 (H3N2)-like virus A/HK/1985/2009, for individuals with RT-PCR-confirmed pandemic H1N1 (left) or seasonal H3N2 infection (right).